FUKUTIN-RELATED PROTEIN ASSOCIATES WITH THE SARCOLEMMAL DYSTROPHIN-GLYCOPROTEIN COMPLEX*

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Running Title: FKRP and the Sarcolemmal DGC

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   Mutations in fukutin-related protein (FKRP) give rise to mild and more severe forms of muscular dystrophy. FKRP patients have reduced glycosylation of the extracellular protein dystroglycan and FKRP itself shows sequence similarity to glycosyltransferases, implicating FKRP in the processing of dystroglycan. However, FKRP localization is controversial and no FKRP complexes are known, so any FKRP-dystroglycan link remains elusive. Here, we demonstrate a novel FKRP localization *in vivo*: in mouse, both endogenous and recombinant FKRP are present at the sarcolemma. Biochemical analyses revealed that mouse muscle FKRP and dystroglycan co-enrich and co-fractionate, indicating that FKRP coexists with dystroglycan in the native dystrophin-glycoprotein complex. Furthermore, FKRP sedimentation shifts with dystroglycan in disease models involving the dystrophin-glycoprotein complex and sarcolemmal FKRP immunofluorescence mirrors that of dystroglycan in muscular dystrophy mice, suggesting that FKRP localization may be mediated by dystroglycan. These data offer the first evidence of an FKRP complex in muscle, and suggest that FKRP may influence the glycosylation status of dystroglycan from within the sarcolemmal dystrophin-glycoprotein complex.

   Mutations in FKRP[1] lead to the allelic muscular dystrophies, MDC1C and LGMD2I, characterized by progressive muscle weakness with variable heart, respiratory and brain involvement (1-3). Whereas the specific function of FKRP is unclear, FKRP and its closest known homolog fukutin share sequence homology with phosphoryl ligand transferases and contain DxD domains common to some glycosyltransferases (4,5). In addition, FKRP-associated muscular dystrophies fall into a growing family of "dystroglycanopathies," which exhibit reduced glycosylation of membrane-associated αDG (6). Extracellular αDG and the transmembrane-spanning βDG bind to dystrophin, sarcoglycans and other proteins to form the dystrophin-glycoprotein complex (DGC), which serves as a critical structural link between the cell cytoskeleton, the sarcolemma and the extracellular basement membrane. αDG glycans, detected by antibody IIH6, mediate the interaction between the DGC and extracellular matrix proteins that contain laminin LG domains. Therefore, reduced αDG glycosylation may weaken the cell to matrix link, increasing structural instability and disease (6).

   Previous studies have examined the localization of FKRP in a spectrum of cultured cells (e.g. COS7, SH-SY5Y, C2C12). The variable results suggested FKRP is a resident of the Golgi, the rER, or perinuclear regions (7-11). In this study, we have examined FKRP protein complexes and their location in skeletal muscle of wild-type and dystrophic mice. We report that FKRP is localized at the muscle sarcolemma and that it co-fractionates with the DGC. Furthermore, disruption of the DGC (by alkaline treatment or genetic deletion) revealed that FKRP sedimentation and localization are dependent on the DGC. Overall, these data suggest that FKRP associates with the sarcolemmal DGC, and that it plays a unique role in dystroglycanopathy muscle disease.

Experimental Procedures

Reagents- Standard laboratory chemicals were used (Fisher, Sigma, Roche, BioRad). Digitonin was special-grade, water soluble (Biosynth AG). Primary antibodies IIH6 αDG, Rbt 83 βDG, and 21B5 γSG have been described (12-14).

MANDRA1 dystrophin (Hybridoma Bank, U. Iowa), heparin sulfate proteoglycan (perlecan, Chemicon), FLAG (Sigma), and HA.11 (Covance) antibodies were used. Custom rabbit polyclonal antisera against mouse FKRP C-terminus and FKRP 176-189 peptides (Suppl. Table 1) were developed (Sigma). Antisera were affinity purified using peptide or recombinant FKRP. FKRP 176-189 purifications were pooled and dialyzed.

Mice- Mouse procedures were ACURF approved. C57BL/6 (Jax 000664), mdx (dystrophin-deficient, Jax 001801), MCK-Cre x T30 (floxed DG excision, MCK DG)(15), αSG null (16), myd (LARGE-deficient, Jax 000300)(17), dysferlin-null (18), 129sve (Taconic), and C57BL/10 (Jax 000665) strains were used. Skeletal muscle from 5 - 24 wk mixed gender mice was frozen for immunofluorescence. Mice were 8 - 30 wk old for biochemical analyses.

Viruses- Mouse FKRP or fukutin cDNAs (Open Biosystems) were used to create 3' 2xFLAG (CFLAG2FKRP) or 3xHA- (fukutin3xHA) tagged constructs (Suppl. Table 2). cDNAs were sequenced (U. Iowa DNA Facility) and cloned into pacAd5CMV shuttle for virus generation (U. Iowa Gene Transfer Vector Core)(19). Five to 7-day old C57BL/6 pups were injected in tibialis anterior or calf muscles with 1 μl virus (2 - 9 x 10^{10} pfu/ml) mixed in 9 μl saline (20).

Biochemistry- DGC enrichment was adapted from previous protocols (14); for buffer constituents, see Suppl. Table 3. Skeletal muscle (3 g) was solubilized and separated at 142,000xg. Supernatants were enriched with WGA-agarose (Vector Laboratories). Pooled WGA elutions were adjusted to 50 mM NaCl, applied to DEAE resin (Whatman), washed and eluted with 100, 150 (Fig. 2 only), 500 and 750 mM NaCl. Elutions (150 or 500 mM) were loaded onto 10 - 30% sucrose gradients (Biocomp Instruments) and sedimented at 220,000xg for 2 hr; 14 fractions were collected. For alkaline experiments (21), DEAE elutions were titrated to pH7.4 or pH11, incubated for 1 hr (22°C), and sedimented on pH7.4 or pH11 sucrose gradients. SDS-PAGE (3 - 15%) and Western blotting on Immobilon-P PVDF (Millipore) were adapted from standard protocols (22). Blocking and antibody incubations were done in TBS-T + 5% milk, low salt TBS-T (75 mM NaCl) + 5% milk (FKRP C-term) or + 3% BSA (FKRP 176-189). HRP-conjugated secondary antibodies were used (Chemicon, Roche). Chemiluminescence (Pierce) was digitally detected (Alpha Innotech).

Immunofluorescence- 7 μm cryosections were incubated overnight in primary antibody (4°C) and for 45 min in Cy3- (Jackson Immunoresearch) or Alexa Fluor 488- (Molecular Probes) conjugated secondary antibodies plus DAPI nuclear stain (Sigma), similar to previous procedures (14). For Fig. 1, sections were fixed and permeabilized (2 % PFA, 0.2 % TX100); for peptide competition, antibody and 1 mg/ml FKRP C-term or control peptide (Suppl. Table 1) were incubated 1 hr and applied to slide. Images were taken at 60x (BioRad MRC600 or Olympus BX61 confocal), 40x or 20x (Leica DMRXA) magnification. Image parameters were identical for 20x and 40x pictures of the same protein in the same panel.

Analyses and Digital Images- Western blot band intensity (area under peak) was autodetected (AlphaEase FC, Alpha Innotech), normalized to the maximum signal for each blot, and plotted versus sucrose fraction number (Microsoft Excel). Western blot and fluorescence images were adjusted for size and signal strength in Photoshop (Adobe). Adjustments were applied equally to the entire image; images using the same primary antibody in the same panel were adjusted identically.

RESULTS

FKRP is a putative glycosyltransferase thought to function in αDG post-translational modification. To begin to elucidate the role of FKRP in skeletal muscle, we tested mouse C57BL/6 quadriceps for FKRP expression using antibodies against mouse FKRP C-terminal (a.a. 481-494) or internal (a.a. 176-189) epitopes. Native FKRP immunofluorescence surrounded individual muscle fibers in a pattern consistent with sarcolemmal staining, and was specific for the FKRP epitope as incubation with antigen peptide eliminated surface signal (Fig. 1A). Native FKRP was also detected at the sarcolemma in longitudinal muscle sections (Fig. 1B). FKRP signal was robust at neuromuscular junctions (Suppl. Fig. 1); however, whether FKRP is targeted to this location or enhanced staining simply reflects additional membrane surface at the end plate is unclear. The FKRP sarcolemmal

staining pattern was reproducibly observed for the C-terminal (Fig. 1) and internal FKRP epitopes (Suppl. Fig. 1), although C-terminal detection was typically stronger. FKRP expression in quadriceps was representative of all muscles tested (hamstring, tibialis anterior, gastrocnemius and soleus; data not shown).

To confirm the presence of FKRP at the plasma membrane, we infected C57BL/6 muscle with viral particles encoding C-terminal fusion tag constructs for FKRP or its closest homolog, fukutin. At 4 weeks post-injection, recombinant FKRP expression was clearly apparent at the sarcolemma in gastrocnemius muscle using anti-FKRP (4 week post-injection, Suppl. Fig. 2A) or anti-FLAG antibodies (data not shown). Intracellular punctate staining showing some overlap with Golgi marker GM130 (data not shown) was observed, but was typically restricted to highly overexpressing fibers. Similar results were obtained with tibialis anterior muscle, at 4 and 6 weeks post-injection (data not shown). In contrast, exogenous fukutin expression at the sarcolemma was minimal, with the majority dispersed throughout infected fibers (Suppl. Fig. 2B). Therefore, recombinant FKRP localization at the sarcolemma is not an artifact of viral protein overexpression, and FKRP and fukutin likely have distinct functions.

The DGC is stably expressed at the sarcolemma and can be purified via multistep biochemical enrichment (12). As we detected FKRP at the membrane surface and this protein is implicated in αDG function, we hypothesized that FKRP may associate with the DGC. To test this, we assessed co-enrichment of FKRP with the DGC in C57BL/6 skeletal muscle. Like the DGC, a ~50kDa FKRP signal was enriched following WGA and ion exchange chromatography (Fig. 2A). When the sample was sedimented by sucrose-gradient fractionation, FKRP expression overlapped with that of all DGC components tested (dystrophin, γSG, α- and βDG; Fig. 2B,C, left) and FKRP and DG peak signals intensities were consistently detected in the same or adjacent fractions demonstrating cosedimentation (n=3). When DGC enriched samples were alkaline treated to disrupt pH-dependent protein binding (21), FKRP and DGC components shifted, by varying degrees, to lighter fractions compared to pH7.4 (Fig. 2B,C, right), indicating partial

disruption of the complex. Dystrophin and γSG were predominately expressed in fractions 8 to 11 and fractions 6 to 10, respectively, whereas αDG, βDG, and FKRP were primarily concentrated in fractions 4 through 8 (96.7 ± 1.9% FKRP vs 94.2 ± 2.2% βDG signal in fractions 4-8, n=3). Protein peaks in the sedimentation profile were detected in the same (n=1) or adjacent fractions (n=2). The shift in FKRP sedimentation most closely mimics that of DG suggesting that FKRP may link to the DGC via the DG subcomplex. However, as there is still some partial overlap with other DGC components, these experiments do not exclude the possibility of other FKRP – DGC interactions. Overall, these data suggest that FKRP is a novel protein that associates with the DGC.

As FKRP associates with the DGC, we speculated that FKRP expression or localization might be altered in dystrophic mouse models in which DGC components are deleted. We probed for FKRP immunofluorescence in dystrophin-deficient (mdx), striated muscle DG-deficient (MCK DG), αSG-null, and αDG-hypoglycosylation (LARGE^{myd}) mice relative to wild-type and non-DGC dystrophic (dysferlin) mice. As expected, FKRP was localized at the muscle sarcolemma in wild-type and non-DGC dystrophic muscle (Fig. 3). Uniform FKRP expression was also observed in αSG- (albeit reduced) and LARGE-deficient muscle, indicating that neither the sarcoglycan complex nor αDG LARGE-dependent glycosylation is required for FKRP localization *per se*. In contrast, FKRP sarcolemmal staining was patchy or absent in dystrophin- or DG-deficient models. Notably, the few muscle fibers with FKRP expression were also positive for α- and βDG (Fig. 3, see asterisks), suggesting that the sarcolemmal localization of FKRP is mediated by its interaction with DG.

An association between FKRP and DG is further implied by co-sedimentation analysis of dystrophic mouse muscle. Both FKRP and DG (α- and βDG) shift to lighter fractions in dystrophin-deficient (mdx) muscle, where the DGC complex is incomplete (Fig. 4A). Similarly, a loss of αSG shifts these proteins to smaller molecular-weight fractions (Fig. 4B). In both cases, FKRP expression appeared to be reduced. Genetic deletion of dystrophin or sarcoglycans reduces

DGC components at the sarcolemma, presumably by destabilizing the complex, and in microsomal membrane preparations from these mice, the expression of DG and FKRP was also reduced (data not shown). Overall, these findings suggest that maintenance of FKRP at the membrane requires DG. In contrast, in non-DGC related dysferlin null muscle (Fig. 4B) and in αDG hypoglycosylation myd muscle (data not shown), FKRP sedimentation was unchanged; it continued to co-migrate with both αDG and βDG. FKRP and DG showed strong co-fractionation in all models tested with only slight differences in distribution or location of peak signal, which may be attributed to experimental variation or a mild preference for FKRP to interact with some subpopulation of DG complexes. Combined, these data reveal a strong and consistent correlation between FKRP sarcolemmal expression and the presence of DG. Although any causative link between the loss of sarcolemmal FKRP and the concomitant loss of DG in these mouse models is currently unknown, it is possible that the absence of FKRP may contribute to pathogenesis in these dystrophies.

DISCUSSION

Our *in vivo* data provide the first indication that FKRP is localized to the sarcolemma in intact skeletal muscle. Previous studies have suggested that FKRP expression is restricted to Golgi, perinuclear, or rough ER domains, although most of this work was done in cultured cells and may be subject to limitations of such *in vitro* systems (7-11). We have likewise observed perinuclear staining of recombinant and native FKRP in various cultured cells using the FKRP C-terminal antibody described here (data not shown). In muscle sections, FKRP signal was reported to be perinuclear (possibly rER) or concentrated to granular structures inside muscle fibers (7,9,11). Although our data conflict with these previous findings, the fact that we detected sarcolemmal staining of native FKRP with antibodies directed against two distinct FKRP epitopes, and confirmed membrane localization of FKRP (but not fukutin) using recombinant fusion proteins, provides strong support for the targeting of FKRP to the sarcolemma. We detected some recombinant FKRP intracellularly, but it is unclear if this is functional protein or an artifact of protein overexpression. The differences between results obtained from cell culture systems versus those from muscle tissue suggest that the sarcolemmal localization of FKRP may depend on tissue-specific (perhaps basement membrane) signals that are absent in cultured cells.

Cell surface and extracellular localization has been described for several glycosyltransferases and related enzymes (23-27). Localization can be tissue-specific, may require proteolysis, and may be regulated by mechanisms such as alternative splicing or putative RNA editing (24,26,27). In this study, native skeletal muscle FKRP was detected as a smaller ~50 kDa protein in skeletal muscle (predicted core protein ~55 kDa). As the entire FKRP open reading frame is contained within one exon, alternative splicing is unlikely to regulate protein size. However, motif analysis (28) of the protein sequence detects putative signal peptide and protease recognition sites that could potentially generate ~50 kDa FKRP.

The DGC, including dystrophin, DG and sarcoglycans, resides at the plasma membrane, anchoring the extracellular basement membrane and cell surface to the cytoskeleton and acting as a scaffold for other proteins (e.g. nNOS, Grb2)(6). We have demonstrated co-enrichment and co-fractionation of FKRP with the DGC via an FKRP association with DG, but not dystrophin or sarcoglycans. While the putative glycosyltransferase LARGE has been shown to bind DG (20), its interaction is believed to be strictly intracellular and transitory as DG passes through the glycosylation pathway. In contrast, our data suggest that the FKRP-DGC association represents a stable, mature complex that is not disrupted by high salt or alkaline conditions. Our finding that FKRP is disrupted in mouse models of muscular dystrophy in which DG is destabilized or lost further suggests that FKRP localization and association may depend on DG. Interestingly, a recent clinical study has identified a novel FKRP mutation in 2 patients. These patients have reduced sarcolemmal DGC proteins rather than a selective loss of αDG glycosylation (29). Therefore, it is possible that FKRP and DGC expression are mutually co-dependent.

Although the precise activity of FKRP remains elusive, patient data support a role in the αDG glycosylation pathway. Although we did not detect native FKRP in intracellular compartments

of skeletal muscle, it is possible that FKRP is enzymatically active as the DGC complex is assembled and processed. Alternatively, FKRP could regulate glycan modification, protein interactions, transit time through internal compartments, or DGC stability. FKRP as a molecular chaperone for DG processing and/or targeting −or *vice versa*− is an intriguing possibility. Whether FKRP activity is required at the sarcolemma for normal muscle function is unclear; however, known glycosyltransferases may possess enzymatic or lectin binding activity at the cell surface (23-25,30). Overall, our data indicate that FKRP is present at the muscle cell surface, associates with the DGC and may have a unique role in the αDG processing pathway.

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FOOTNOTES

[1]Abbreviations: FKRP, fukutin-related protein; MDC1C, congenital muscular dystrophy 1C; LGMD2I, limb girdle muscular dystrophy 2I; αDG, α-dystroglycan; βDG, β-dystroglycan; DGC, dystrophin-glycoprotein complex; DG, dystroglycan; α,β,δ or γSG, α-, β-, δ- or γ-sarcoglycan; KLH, keyhole limpet hemacyanin; BSA, bovine serum albumin; MCK, muscle creatine kinase promoter; LARGE, like-glycosyltransferase; HA, hemagglutinin epitope; WGA, wheat germ agglutinin; DEAE, DE52 diethylaminoethyl cellulose; PVDF, polyvinylidene fluoride; TBS-T, Tris buffered saline + 0.1% Tween 20; HRP, horse radish peroxidase; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; PFA, paraformaldehyde; TX100, triton-X 100; rER, rough ER; nNOS, neuronal nitric oxide synthase; Grb2, growth factor receptor bound protein 2.

FIGURE LEGENDS

Fig. 1. FKRP is expressed at the sarcolemma in skeletal muscle. Confocal immunofluorescence images of C57BL/6 quadriceps muscle; 60x magnification, scale bar 25 µm applies to all images. A, Peptide competition experiment. Control or FKRP peptide was incubated with FKRP antibody (FKRP C-term) before application to postfixed, permeabilized cryosections. Prominent FKRP sarcolemmal staining was specifically inhibited by the FKRP antigen but not control peptide (αDG staining was unaffected by

peptide, data not shown). B, FKRP (C-term) was detected at the sarcolemma in longitudinal muscle sections (no signal in the absence of FKRP antibody, data not shown).

Fig. 2. FKRP and DG co-enrich and co-sediment, even after alkaline treatment. A, For DGC preparation, solubilized C57BL/6 skeletal muscle was enriched with WGA and DEAE chromatography. WGA elutions (elu1 and 2) and DEAE samples (void, wash and 100, 150, 500, and 750 mM NaCl elutions) were collected and probed by Western blot for αDG (αDG IIH6) and FKRP C-term. FKRP (~50 kDa, black arrow) co-enriches with DG. Molecular weight markers are shown in kDa. B, DEAE elutions were incubated at normal (pH7.4) or alkaline pH (pH11.0), then separated on 10 - 30% sucrose gradients. Gradient fractions (1=top, 14=bottom) were blotted with antibodies against dystrophin, γSG, βDG (grey arrow; reprobe of γSG blots), glycosylated αDG IIH6 (αDG) or FKRP (C-term). C, Band intensities were normalized (Area norm) and plotted against gradient fraction number. Data are representative of 3 experiments.

Fig. 3. FKRP sarcolemmal expression is lost in mouse models of DG protein deficiency. Serial cryosections of quadriceps muscle from wild-type and dystrophic mouse models were processed with antibodies against FKRP, βDG, glycosylated αDG (IIH6), and perlecan. Images were captured at 40x magnification, scale bar 50 μm. Asterisks show same fibers in serial sections; note that FKRP is expressed in the same fibers as DG. Mouse models: mdx, dystrophin mutant; MCK DG, MCK-cre excision of floxed DG; αSG, αSG-null; myd, LARGE mutant; dysferlin, dysferlin-null.

Fig. 4. FKRP expression and cosedimentation mimic those of DG in dystrophic mouse models. DGC enrichment and sucrose gradient fractionation were performed as in Fig. 2. Gradient fractions were blotted for FKRP (C-term), glycosylated αDG (IIH6), and βDG. A, C57BL/6 and mdx muscle. B, Dysferlin-null (non-DGC muscular dystrophy) and αSG-null muscle. Data are representative of 2 to 5 experiments.